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Effects of hypoxia–reoxygenation stimuli on renal redox status and nuclear factor erythroid 2-related factor 2 pathway in sickle cell SAD mice

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Abstract

Hypoxia–reoxygenation (H/R) stress is known to increase oxidative stress in transgenic sickle mice and can cause organ failure. Here we described the effects of H/R on nuclear factor erythroid 2-related factor 2 (Nrf2) as a putative regulator of redox status in the kidneys of SAD mice investigating Nrf2-regulated antioxidant enzymes. Transgenic SAD mice and healthy C57Bl/6J mice were exposed to 4 h of hypoxia followed by various times of reoxygenation at ambient air (2 or 6 h). Regardless of the conditions (i.e. normoxia or H/R), SAD mice expressed higher renal oxidative stress levels. Nuclear Nrf2 protein expression decreased after 2 h post-hypoxia only in the medulla region of the kidney and only in SAD mice. Simultaneously, haem oxygenase transcripts were affected by H/R stimulus with a significant enhancement after 2 h post-hypoxia. Similarly, hypoxia inducible factor-1α staining increased after 2 h post-hypoxia in SAD mice in both cortex and medulla areas. Our data confirm that the kidneys are organs that are particularly sensitive to H/R stimuli in sickle cell SAD mice. Also, these results suggest an effect of the duration of recovery period (short *vs.* long) and specific responses according to kidney areas, medulla *vs.* cortex, on Nrf2 expression in response to H/R stimuli in SAD mice.

KEYWORDS

antioxidant response, hypoxia-inducible factor-1α, renal injury

1 | INTRODUCTION

Sickle cell disease (SCD) is a genetic haemoglobinopathy characterized by anaemia, haemolysis (Kato, Steinberg, & Gladwin, 2017), impaired red blood cell rheology (Connes et al., 2014), enhanced inflammation (Rees, Williams, & Gladwin, 2010), endothelial dysfunction (Wood, Hsu, & Gladwin, 2008) and oxidative stress (Chirico & Pialoux, 2012), which participate in the development of acute vaso-occlusive-like events in various organs (Belcher, Beckman, Balla, Balla, & Vercellotti, 2010). These vascular obstructions cause intermittent cessation of blood flow to tissues and subsequent ischaemia, followed by reoxygenation phases resulting in increased reactive oxygen species (ROS) production leading to damage of tissues (Chirico & Pialoux, 2012; Hebbel, 2014; Nur, Biemond, Otten, Brandjes, & Schnog, 2011; Osarogiagbon et al., 2000), such as those located at the kidney level (Ataga, Derebail, & Archer, 2014). The occurrence of oxidant-mediated injury via ischaemia–reperfusion (I/R) cycles has been demonstrated in more and less severe transgenic sickle cell mouse models (Aufradet et al., 2013; Aufradet et al., 2014; Juncos et al., 2010; Kaul & Hebbel, 2000; Osarogiagbon et al., 2000). In this context, the involvement of

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the xanthine oxidase/xanthine dehydrogenase pathway has been previously reported (Kaul, Liu, Chang, Nagel, & Fabry, 2004; Osarogiagbon et al., 2000).

Nuclear factor erythroid 2-related factor 2 (Nrf2), a basic leucine zipper transcription factor, is one of the main regulators of antioxidant production (Lee et al., 2005). Under normoxic conditions Nrf2 is rapidly degraded by Kelch-like ECH-associated protein 1 (Keap-1)-mediated ubiquitination in the cytoplasm (Zhang, Lo, Cross, Templeton, & Hannink, 2004). In contrast, in situations in which oxidative stress is enhanced (Kang, Lee, & Kim, 2005; Leonard et al., 2006; Sangokoya, Telen, & Chi, 2010), there is a disruption of the cytoplasmic complex Nrf2–Keap1, which leads to nuclear translocation of Nrf2 where it heterodimerizes with members of a small family of protein known as musculoaponeurotic fibrosarcoma (Maf) (Bryan, Olayanju, Goldring, & Park, 2013; Jaiswal, 2004). Another recent regulatory pathway of Nrf2 under hypoxia has been reported by Baba, Morimoto, and Imaoka (2013) and involves seven in absentia homolog2 (Siah2), a potent RING finger E3 ubiquitin ligase that regulates hypoxia-activated pathways (Nakayama et al., 2004). Once activated, Nrf2 binds to the antioxidant response element on the promoter regions of several key antioxidant genes, notably haem oxgenase-1 (HO-1) (Baba et al., 2013; Ghosh et al., 2011; Jaiswal, 2004). HO-1 is a cytoprotective enzyme involved in the catabolism of haem, a major source of ROS in SCD via its conversion to carbon monoxide (CO), biliverdin and ferritin (Choi & Alam, 1996). Recently, Belcher et al. (2018) reported a key role of HO-1 in the inhibition of microvascular stasis in a transgenic sickle mouse model infused with haemopexin and haptoglobin. HO-1 has also been shown to be modulated by the α-subunit of hypoxia-inducible factor 1α (HIF-1α) (Lee et al., 1997; Yang & Zou, 2001).

Similarly to sickle cell patients (McPherson Yee et al., 2011; Ranque et al., 2014), transgenic SAD mice are exposed to repeated kidney subclinical vaso-occlusions which may promote the development of glomerulopathy (De Paepe & Trudel, 1994; Sabaa et al., 2008), and ultimately lead to renal failure. While the activation and up-regulation of Nrf2 and Nrf2-dependent antioxidant gene expression in the kidneys is well described in models of I/R injury (Leonard et al., 2006), the involvement of Nrf2 in mediating antioxidant responses to hypoxia–reoxygenation (H/R) stimuli in the kidneys of transgenic SAD mouse models has never been investigated. The aim of the present study was to describe the effects of H/R stimuli on the regulation of the renal redox status and on the Nrf2 pathway in a sickle cell SAD mouse model to gain further insight into the role of oxidative stress in the development of chronic kidney damage in sickle cell disease.

New Findings

- **What is the central question of this study?**
  What are the effects of repeated subclinical vaso-occlusions on nuclear factor erythroid 2 related factor 2 (Nrf2) and oxidative stress balance regulation in the kidney of transgenic SAD mice?
- **What is the main finding and its importance?**
  In response to hypoxia–reoxygenation, nuclear Nrf2 protein expression decreased in the kidney of SAD mice while haem oxygenase transcripts were increased. This suggest that in SAD mice, other transcription factors than Nrf2 could be involved in renal antioxidant gene regulation in response to hypoxia–reoxygenation.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

The guidelines from the French Ministry of Agriculture for experimental procedures and the Institute for Laboratory Animal Research (National Academy of Sciences, USA) were followed and the protocol was approved by the regional animal care committee (Rhône-Alpes, France) with approval number DR2013-46.

2.2 | Mice

Healthy (C57Bl/6J) and sickle cell SAD mice were used in this study and were raised on standard mouse chow. Mice received water and food *ad libitum* and were kept under a 12 h–12 h day–night rhythm. SAD mice, originally generated by the team of Dr Trudel and Prof. Beuzard, are hemizygous for a modified human sickle haemoglobin, HbSAD ($\alpha_2{}^{human}\beta_2{}^{SAD}$) that includes three sickle mutations: D-punjab (β^{121}Gln), Antilles (β^{23}Ile) and β^s (β^6Val). The coexpression of the β^{SAD} globin gene and of the human α globin gene resulted in 19% HbSAD in the red blood cells (RBCs) of transgenic SAD mice (Trudel et al., 1991). This model is particularly interesting since it induces experimental vaso-occlusive events in response to controlled H/R. Organ injuries induced by H/R stimuli closely mimic an acute hypoxia-induced vaso-occlusive event of human SCD (de Franceschi et al., 2003; De Franceschi, Brugnara, Rouyer-Fessard, Jouault, & Beuzard, 1999; Trudel et al., 1994) more than chronic damage since analysis of the kidney was performed only a few hours after the exposure. Also, we previously reported that such a protocol, 4 h of vaso-occlusive hypoxic stress followed by 2 or 6 h of reoxygenation in ambient air, induced pulmonary endothelial activation and increased hemolysis, cardiac oxidative stress and inflammation (tumour necrosis factor-α, interleukin-6) (Aufradet et al., 2013, 2014; Charrin et al., 2015). Normal control mice (C57) had the same genetic background as SAD mice (i.e. C57Bl/6J) (Charles River, L'Arbresle, France). Mean age was 11.1 and 10.6 weeks for C57 and SAD mice, respectively.

2.3 | Hypoxia–reoxygenation

Thirty-five SAD mice and 36 C57 mice were randomly divided into three groups of each strain, one normoxic ($C57_{Nx}$, SAD_{Nx}) and two hypoxic groups, which were exposed to hypoxia with 6.5% O_2 and

93.5% N_2 for 4 h followed by 2 h ($C57_{2h}$, SAD_{2h}) or 6 h ($C57_{6h}$, SAD_{6h}) of reoxygenation with room air. To induce hypoxia, mice were placed in their own cage in a chamber (Biospherix, Redfield, NJ, USA) equipped with an oxygen sensor system (Pro-ox, BioSpherix, Parish, NY, USA) controlling the pure N_2 gas distribution (Nitrogen 4.5, Linde Gaz s.a, St Priest, France). The O_2 proportion in the chamber was progressively decreased from 21% to 6.5% after the entrance of the mice and then adjusted to supply 6.5% for the total duration of the hypoxia period. These H/R paradigms have been shown to stimulate factors involved in vaso-occlusive events (Aufradet et al., 2013, 2014).

2.4 | Tissue sampling

At the end of the protocol mice were killed by an injection of dolethal (400 μg (g body weight)$^{-1}$). Both whole kidneys were quickly isolated and one, for histology and immunohistology analyses, was fixed in 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) in a 0.1 mol l^{-1} phosphate-buffered saline (PBS; Sigma-Aldrich) for 2 h followed by 48 h in 25% sucrose (Sigma-Aldrich) for cryoprotection and finally frozen in isopentane (−40°C, VWR, Briare, France) before storage at −80°C. The other kidney was immediately immersed in liquid nitrogen, and then stored at −80°C for RT-qPCR and oxidative stress analyses. It was then ground in liquid nitrogen using an RNase-free piston pellet and separated in two tubes for RT-qPCR and biochemical analyses. For oxidative stress analyses the kidney was homogenized (10%, w/v) in 1×PBS + 0.5 mM EDTA in ice.

2.5 | Real Time-quantitative polymerase chain reaction

2.5.1 | HO-1, G6PD, glutamate cysteine ligase and aldehyde dehydrogenase

Total RNAs were isolated using the RNeasy Plus Micro kit (Qiagen, Courtaboeuf, France). RNAs were retro-transcribed into cDNA using Superscript II Reverse Transcriptase and qPCR was carried out in triplicate on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Reaction mixtures had a final volume of 10 μl, consisting of 2 μl of cDNA, 5 μl of LightCycler® 480 SYBR Green I Master and 0.5 μM primers. After initial denaturation, amplification was performed at 95°C (10 s), 60°C (5 s) and 72°C (10 s) for 45 cycles. Calculation of relative expression was determined by the Bio-Rad CFX Manager™ software and fold change was normalized as normalized relative quantity (or $\Delta\Delta C_q$) for each series, as:

$$\mathrm{NRQ} = \frac{2\Delta^{C_q\mathrm{T}-C_q\mathrm{Cal}}}{2\Delta^{C_q\mathrm{R}-C_q\mathrm{Cal}}}$$

where T is the target sample, Cal the calibrator value (i.e. the mean of all sample Cqs of the serie) and R is the housekeeping gene *Cyclophilin A* for mouse. Log2-NRQ values were used to perform statistical analyses. The *Mus musculus* primer pairs used in this study were: *haem oxygenase-1* (HO-1; NM_007393.3) left 5′-GTC-AAG-CAC-AGG-GTG-ACA-GA-3′, right 5′-ATC-ACC-TGC-AGC-TCC-TCA-AA-3′ (77 bp); *glucose-6-phosphate dehydrogenase* (G6PD, Z11911.1) left 5′-ACG-ACA-TCC-GAA-AGC-AGA-GT-3′, right 5′-CAT-AGG-AAT-TAC-GGG-CAA-AGA-3′(90 bp); *glutamate cysteine ligase* (NM_008129.3) left 5′-TGA-CTC-ACA-ATG-ACC-CGA-AAA-3′, right 5′-TCA-ATG-TCA-GGG-ATG-CTT-TCT-3′ (79 bp); *aldehyde dehydrogenase1* (NM_011921.2) left 5′-CAA-GCT-GGC-TGA-CTT-AAT-GGA-3′, right 5′-GAC-TTT-CCC-AGC-ATT-CAT-CG-3′ (73 bp).

2.5.2 | ET-1, iNOS, VEGF-A and EPO

Total mRNA from the kidneys was isolated using Tri Reagent LS (Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions. Then, the RNA solution extracted was purified with the Ambion® TURBO DNA-*free*™ (Ambion, Austin, TX, USA) and standardized at 80 ng μl^{-1}. Total RNA was converted to cDNA by reverse transcription as follows. A reaction mixture containing 1000 ng of RNA was mixed with 1μg of $Poly(T)_{15}$ and 100 units (U) of reverse transcriptase RNase Hminus (Promega, Madison, WI, USA). A synthetic external and non-homologous poly(A)Standard RNA (SmRNA) was used to calibrate the reverse transcription of mRNAs of biological samples (Morales and Bezin, patent WO2004.092414). Real-time PCR analysis was performed on a lightCycler® System (Roche, Penzberg, Germany) by using the Quantitec SYBR®green PCR kit (Qiagen, Venlo, Netherlands). The results for targeted mRNAs were normalized against the SmRNA. The *Mus musculus* primer pairs used in this study were: *endothelin-1* (ET-1, NM_010104.2) left 5′-CTG-CTG-TTC-GTG-ACT-TTC-CA-3′, right 5′-TCTGCACTCCATTCTCAGCTC-3′ (85 bp); *inducible nitric oxide synthase* (iNOS, NM_010927.3) left 5′-GGG-CTG-TCA-CGG-AGA-TCA-3′, right 5′-CCA-TGA-TGG-TCA-CAT-TCT-GC-3′ (66 bp); *vascular endothelial growth factor-A* (VEGF-A, NM_001025250.3) left 5′-AAAAACGAAAGCGCAAGAAA-3′, right 5′-TTTCTCCGCTCTGAACAAGG-3′ (73 bp); *erythropoietin* (EPO, NM_007942.2) left 5′-TCT-GCG-ACA-GTC-GAG-TTC-TG-3′, right 5′-CTT-CTG-CAC-AAC-CCA-TCG-T-3′ (78 bp).

2.6 | Oxidative stress, enzymatic and non-enzymatic antioxidant activities

A first centrifugation at 1500 *g* for 4 min at 4°C was performed to collect the supernatant for malondialdehyde (MDA) and protein assays. Then, a second centrifugation was done at 12,000 *g* during 10 min at 4°C to collect the remaining supernatant for advanced oxidation protein products (AOPP) assays and measurements of SOD, glutathione peroxidase (GPX) and catalase. These oxidative stress assays are standard procedures in our laboratory (Faes et al., 2012).

AOPP, which reflects protein oxidation, were measured using the semi-automated method as previously described (Pialoux, Brown, Leigh, Friedenreich, & Poulin, 2009). Briefly, concentrations were determined by spectrophotometry on a microplate reader and were calibrated with a chloramine-T solution that absorbs at 340 nm in

the presence of potassium iodide. The absorbance of the reaction was immediately read at 340 nm against a blank containing 200 µl of PBS. AOPP concentrations were expressed as micromoles per litre of chloramines-T equivalents.

Concentrations of MDA as thiobarbituric reactive substances were determined as previously described (Pialoux et al., 2006). The pink chromogen was extracted with *n*-butanol and its absorbance was measured at 532 nm by spectrophotometry using 1,1,3,3-tetraethoxypropan as standard. Although the MDA assay is often considered to be only a moderately sensitive and specific technique, it is still widely used as an oxidative stress marker of lipid peroxidation.

Plasma SOD activity was assayed using the method of Beauchamps and Fridovich (1971), slightly modified by Oberley and Spitz (1984). This activity was determined by measuring the degree of inhibition of the reaction between superoxide radicals, produced by a hypoxanthine–xanthine oxidase system, and nitroblue tetrazolium.

Catalase activity in the plasma was assessed by the method of Johansson and Borg (1988), which used hydrogen peroxide (H_2O_2) as substrate and formaldehyde as standard. Catalase activity was evaluated by the formation rate of formaldehyde.

GPX was determined by the modified method of Paglia and Valentine (1967). For that purpose, the rate of oxidation of NADPH to $NADP^+$ after addition of the glutathione reductase (GR), reduced glutathione (GSH) and NADPH was determined using H_2O_2 as substrate.

2.7 | Immunofluorescence

Cryosections 5 µm thick were prepared for immunolabelling using a NX50 (Microm Microtech, Brignais, France) and stored at −80°C. Before labelling, slides were air dried for 20 min at room temperature. To reduce kidney autofluorescence, sections were incubated in a 70% ethanol–0.1% Sudan Black B (Sigma-Aldrich, cat. no. 199664) solution for 20 min, then washed for 1 min in ethanol 70% and 5 times for 5 min in PBS. Sections were permeabilized for 10 min with PBS–0.5% Triton X-100 (Sigma-Aldrich, cat. no. T8787) and blocked with PBS containing 2% BSA (Roche Diagnostics, cat. no. 10735086001) and 5% donkey serum (Sigma-Aldrich, cat. no. D9663) for 1 h. Labelling was performed with a rabbit polyclonal anti-Nrf2 antibody (Abcam, Cambridge, UK, RU, ab31163) overnight at 4°C and revealed with a Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA, cat. no. 711-165-152) for 2 h at room temperature. On each slide, a negative control with no primary antibody was performed to determine the background level. For nuclear staining, slides were soaked for 10 s in Hoechst solution H 33342 (Sigma-Aldrich, cat. no. B2261) and washed once with PBS before mounting with anti-fading Fluoromount G medium (Interchim, cat. no. FP-483331). Images were acquired at ×40 magnification using an Axio Observer.Z1 microscope (Zeiss, Iéna, Germany) connected to a CoolSNAP HQ2 CCD Camera (Photometrics, Tucson, AZ, USA). For medulla, an average of 2.9 (negative control) and 4.1 (Nrf2 labelling) images chosen randomly were analysed. For the cortex, an average of 2.9 (negative control) and five (Nrf2 labelling) images were analysed.

The percentage of nuclei expressing Nrf2 was determined using the cell counter in ImageJ software (NIH, Bethesda, MD, USA). The quantification was performed blindly by two independent experimenters and averaged. A macro was designed in ImageJ to measure Nrf2 mean fluorescence intensity (MFI). First, a mask was generated using either Nrf2 or Hoechst labelling to select either the total area of Nrf2 expression or the nuclei. Nrf2 MFI was then measured, and the signal was averaged between the fields. Finally, a ratio between the MFI in the sample stained with and without the anti-Nrf2 antibody was calculated for each sample.

2.8 | Histology and immunohistology

Cryosections 10 µm thick were prepared for histology and immunohistology using a NX50 (Microm Microtech France) and tissues were prepared according to conventional procedures. Sections were then stained with haematoxylin and eosin and examined with a light microscope (BX43; Olympus, Tokyo, Japan). Histological analyses were performed by an experienced blinded pathologist.

For immunohistological analyses, after rinsing in PBS, tissue sections were permeabilized in 0.3% Triton X-100 in PBS, then blocked using 1% goat serum for 1.5 h at room temperature. Sections were incubated overnight at 4°C with a rabbit polyclonal anti-HIF-1α (NovusBio, Littleton, CO, USA; cat. no. NB100-449), primary antibody diluted at 1:100. Then, after washing in PBS, sections were incubated with a biotinylated anti-rabbit IgG (Jackson ImmunoResearch Laboratories; cat. no. 106974), secondary antibody diluted at 1:500, for 2.5 h at room temperature. Exposure was performed with the avidin–biotin enzyme complex (Vectastain Elite ABC standard peroxidase kit; Vector Laboratories, Burlingame, CA, USA) and the substrate 3,3′-diaminobenzidine (DAB; Peroxidase Substrate Kit; Vector Laboratories). Specific staining was assured using control slides where the primary antibody was omitted. Images were visualized under a light microscope (Olympus BX43) and captured with a video camera (SC30; Olympus) (×40 magnification). HIF-1α was quantified as the number of HIF-1α positive cells divided by the total number of cells using ImageJ software. At least 5 (for medulla) and 10 (for cortex) fields chosen randomly were analysed.

2.9 | Statistics

All variables were tested for normality and variance homogeneity. Comparisons of the different parameters were done using factorial ANOVA followed by pairwise comparisons for oxidative stress and RT-qPCR analyses and a bilateral Student's *t* test was performed for EPO, ET-1, iNOS, VEGF-A and HIF-1α to compare C57 and SAD mice in normoxia. Statistical significance was determined by P value < 0.05. Analyses were conducted using Statistica (Version 8.0; StatSoft, Tulsa, OK, USA) and GraphPad Prism software (version 6.01; GraphPad Software, Inc., La Jolla, CA, USA) and data were reported as means $\pm$ SD.

TABLE 1 Oxidative stress markers and antioxidant activities under normoxia (Nx) and after 4 h of hypoxia followed by 2 or 6 h reoxygenation in the kidney of healthy C57Bl/6J (C57) and sickle cell SAD mice

	C57			SAD			Group effect
Number of mice/group	Nx	2 h	6 h	Nx	2 h	6 h	
n	12	6	6	6	6	9	
AOPP (μmol l^{-1} of chloramines-T equivalents)	3.7 ± 0.7	3.9 ± 1.3	2.9 ± 1.0	4.3 ± 1.1	4.2 ± 0.5	4.3 ± 1.1	*
MDA (μmol l^{-1})	4.6 ± 2.0	6.2 ± 1.9	6.1 ± 1.4	13.3 ± 7.6††	11.0 ± 4.3	13.6 ± 8.3	***
SOD (μmol l^{-1} min^{-1})	2.9 ± 0.7	3.5 ± 0.5	3.1 ± 0.2	3.8 ± 1.1†	3.9 ± 0.5	3.3 ± 0.4	*
Catalase (μmol l^{-1} min^{-1})	1.9 ± 0.3	2.0 ± 0.4	1.9 ± 0.2	2.8 ± 0.5††	3.3 ± 1.0‡‡,§	2.6 ± 0.4	***
GPX (nmol l^{-1} min^{-1})	9.1 ± 2.6	9.0 ± 1.7	9.2 ± 1.5	10.9 ± 2.7	12.4 ± 2.9	11.2 ± 3.8	**

Values are expressed as means ± SD. AOPP, advanced oxidation protein products; GPX, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$: significant difference between C57 and SAD mice; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$: significant difference between $C57_{Nx}$ *vs.* SAD_{Nx}; $^{\ddagger\ddagger}P < 0.01$: significant difference compared with SAD_{6h}; $^{\S}P = 0.06$: trend compared with SAD_{Nx}.

3 | RESULTS

3.1 | Oxidative stress level in the kidney of SAD mice

At baseline, while AOPP concentrations did not differ between the two groups, SAD mice exhibited significantly higher MDA ($P < 0.01$, Table 1) as compared with C57 mice. We also found a global group effect with significantly greater levels of AOPP and MDA in SAD mice compared with C57 mice. However, H/R stimuli did not significantly change AOPP or MDA levels in either group.

Further, SOD and catalase activities were significantly greater in SAD_{Nx} mice than in healthy $C57_{Nx}$ mice ($P < 0.05$), whereas GPX activity did not differ between the two groups at this time point. A global group effect was also observed for all of these enzymes (SOD, catalase, GPX) with significantly higher enzyme activities in SAD mice than in healthy C57 mice. While we did not find an H/R effect in C57 and SAD mice for SOD and GPX antioxidant activities, catalase activity increased at 2 h post-hypoxia compared with normoxia and decreased after 6 h compared with 2 h post-hypoxia only in SAD mice (+20.7%, $P = 0.06$ and +29.6%, $P < 0.01$, respectively; Table 1). These results showed that in normoxia, renal oxidative stress balance was altered with greater lipid peroxidation and higher antioxidant enzymes suggesting that the SAD kidneys are organs that have increased oxidative stress on a chronic basis.

3.2 | Nuclear Nrf2 protein expression in the kidney

Oxidative stress is tightly regulated by Nrf2. Elevation of ROS triggers the Nrf2 antioxidant response pathway (Kang et al., 2005; Pullarkat, Meng, Tahara, Johnson, & Kalra, 2014) and therefore plays a critical role in protecting various tissues including the kidneys (Lee et al., 2005).

We found a global group effect with significantly greater levels of medullary Nrf2 in the nucleus of SAD mice compared with C57 mice (Figure 1a,c, $P < 0.01$). Further, nuclear Nrf2 expression in the medulla was decreased in SAD mice at 2 h post-hypoxia compared with normoxia ($P < 0.05$) while it did not change in C57 mice regardless of the conditions (i.e. normoxia or H/R) (Figure 1a,c). The down-regulation of nuclear Nrf2 protein expression in the medulla, in SAD mice, after 2 h of reoxygenation may suggest an effect of the duration of reoxygenation. In contrast, we did not find any significant differences of nuclear Nrf2 protein expression in the cortex in both C57 and SAD mice (Figure 1b,d). Those results suggest a specific sensitivity of kidney areas in SAD mice in response to H/R stimuli regarding Nrf2 expression.

3.3 | Nrf2-regulated antioxidant enzymes: HO-1, glutamate cysteine ligase, G6PD and aldehyde dehydrogenase mRNA levels

Renal I/R injury triggered Nrf2 activation, which was found to enhance gene expression of antioxidants and NADPH synthesis enzymes such as glutamate cysteine ligase, a glutathione biosynthesis-related protein, aldehyde dehydrogenase, HO-1 and G6PD, respectively (Leonard et al., 2006; Nezu et al., 2017). In our study, during normoxia and after H/R G6PD, glutamate cysteine ligase and aldehyde dehydrogenase mRNA levels were not affected in either group and no significant inter-group (i.e. SAD *vs.* C57) difference was observed (Figure 1f–h).

In the same way, baseline HO-1 mRNA was not different between $C57_{NX}$ and SAD_{NX}. Surprisingly, compared with normoxia and 6 h post-hypoxia, HO-1 mRNA increased at 2 h post-hypoxia only in SAD mice (+1733% and 572% respectively, $P < 0.001$). Furthermore, HO-1 mRNA was more expressed in SAD_{2h} mice compared with $C57_{2h}$ mice ($P < 0.001$) (Figure 1e). These inconsistent results between HO-1 transcripts and Nrf2 activation may suggest the involvement of other regulatory pathways in response to H/R stimuli.

3.4 | HIF-1α immunostaining and HIF-1α regulated proteins: EPO, VEGF-A, ET-1 and iNOS mRNA

HIF-1α, was reported to be involved in HO-1 gene regulation in the kidneys, and more specifically in the renal medulla, under hypoxia stimuli (Yang & Zou, 2001).

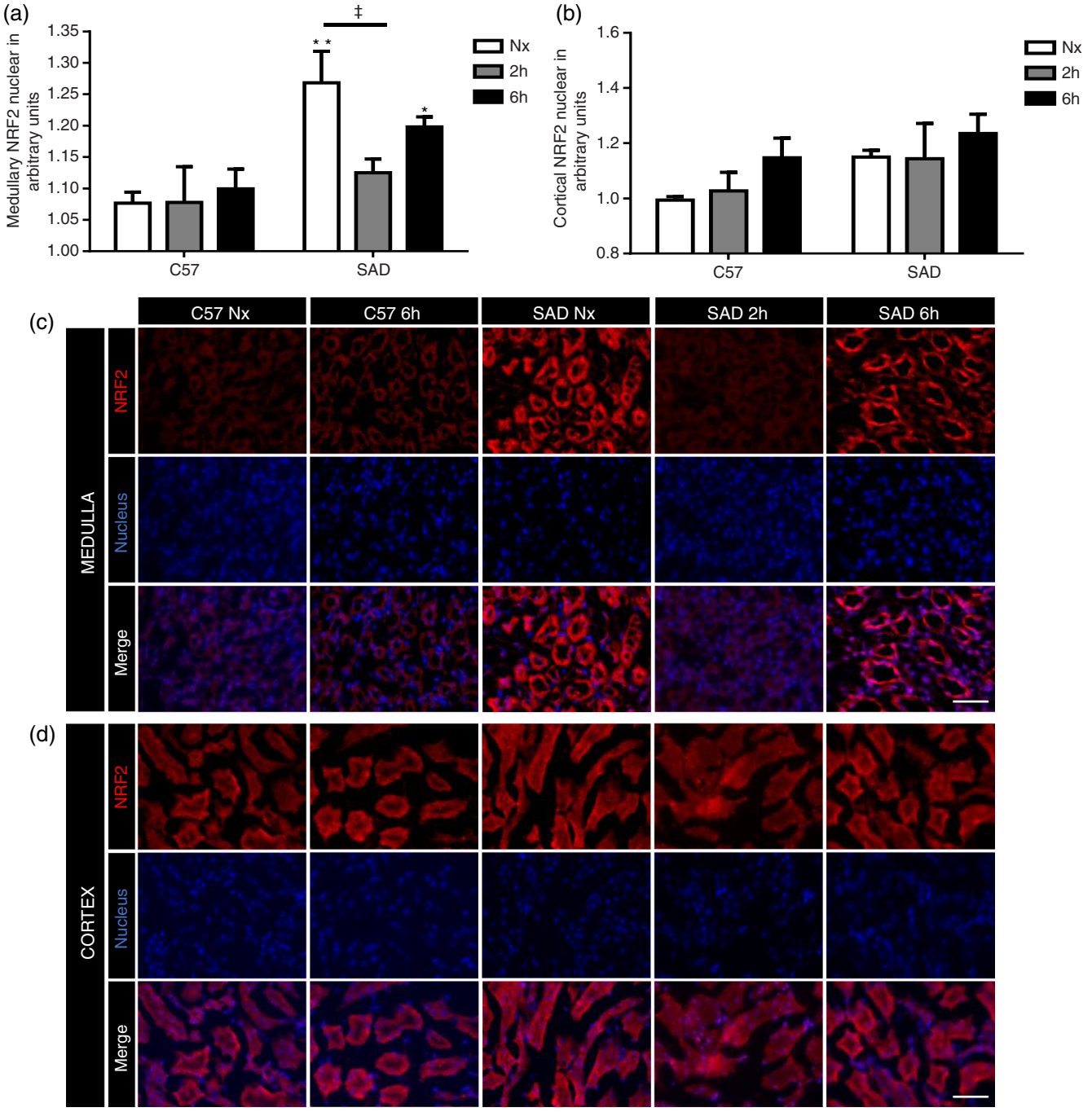

FIGURE 1 Nuclear factor erythroid 2-related factor 2 (Nrf2) expression and Nrf2-regulated antioxidant gene mRNA levels in the kidney of healthy C57Bl/6J (C57) and sickle cell SAD mice under normoxia (Nx) and after 4 h of hypoxia followed by 2 h or 6 h reoxygenation. (a,b) Nuclear Nrf2 protein expression in the medulla (a) and in the cortex (b) of C57 and sickle cell SAD mice in normoxia and after hypoxia–reoxygenation stimuli. Data are expressed in arbitrary units which correspond to the ratio between the mean fluorescence intensity of Nrf2 and the non-specific mean fluorescence intensity. (c,d) Representative images of Nrf2 expression localized in the nucleus (pink) by immunofluorescence on medullary (c) and cortical (d) kidney sections from C57 and SAD mice. Magnification ×40; scale bars 50 μm. (e–h) mRNA levels of haem oxygenase (HO-1) (e), glutamate cysteine ligase (f), glucose 6-phosphate dehydrogenase (G6PD) (g) and aldehyde dehydrogenase (h) expressed as normalized relative quantity (NRQ) in C57 and sickle cell SAD mice in normoxia (white bars) and after hypoxia followed by 2 h (grey bars) or 4 h (dark grey bars) of reoxygenation. $^{*}P < 0.05$, $^{**}P < 0.01$: significant difference between C57 and SAD; $^{\dagger\dagger\dagger}P < 0.001$: significant difference compared with SAD_{2h}; $^{\ddagger}P < 0.05$: significant difference compared with SAD_{Nx}. *n* values per group: C57 Nx and SAD Nx: 3; C57 2 h and SAD 2 h: 3; C57 6 h and SAD 6 h: 4

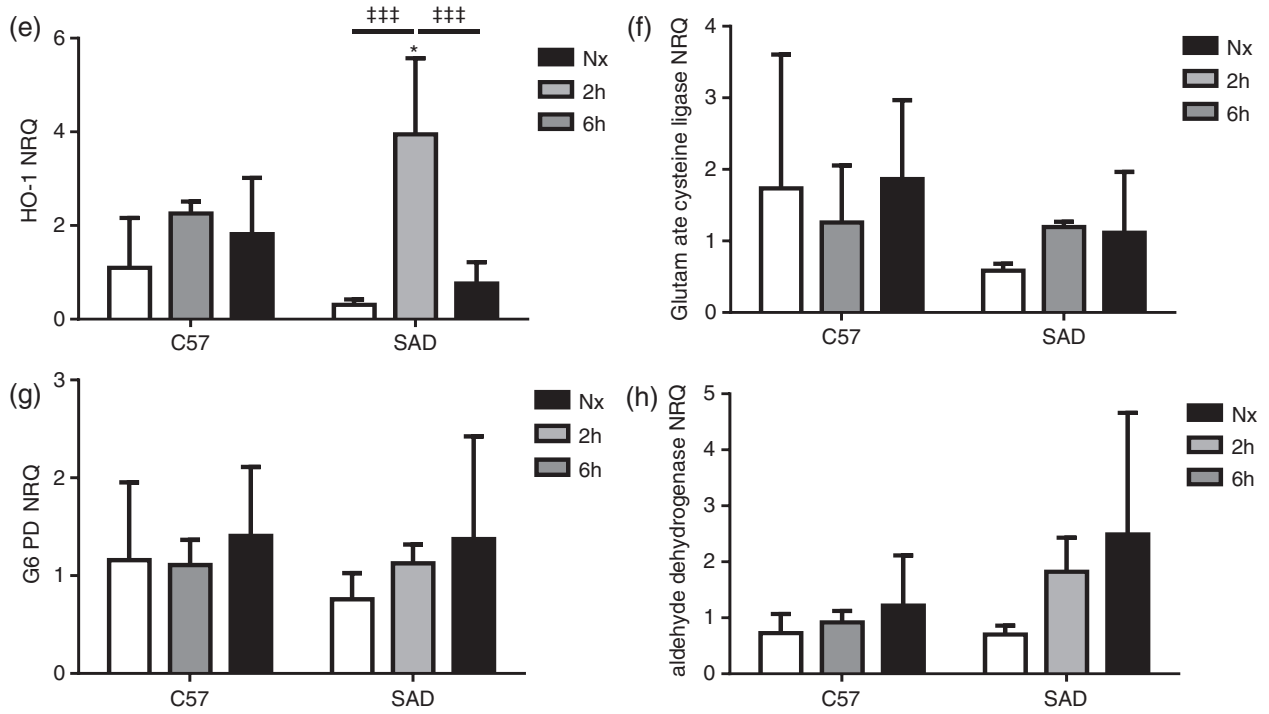

FIGURE 1 Continued

In normoxia, HIF-1α staining in the cortex and medulla of the kidney was markedly more intense in SAD *vs.* C57 control mice ($P < 0.001$, Figure 2a–d). Among the cluster of oxygen-sensitive genes regulated by HIF-1α, SAD mice had higher mRNA expression of iNOS ($P < 0.01$), EPO and ET-1 ($P < 0.05$), and VEGF-A ($P = 0.09$), as compared with C57 mice (Figure 2e–h).

In SAD mice, HIF-1α staining in the cortex and medulla of the kidney increased in response to 2 h post-hypoxia (+70.3%, $P < 0.001$ for cortex, Figure 3b,d) and decreased after 6 h post-hypoxia (−33.9%; $P < 0.01$ for cortex and −45.5% $P < 0.001$ for medulla, Figure 3a–d) as compared with normoxia values. We also found significantly higher levels of HIF-1α after 2 h post-hypoxia *vs.* 6 h post-hypoxia ($P < 0.001$ for medulla and cortex, Figure 3a–d). Overall, in SAD mice, mRNA expression of HIF-1α regulated proteins decreased after 6 h post hypoxia (Figure 3e–h). Therefore, the increased transcriptional activity of HO-1 at 2 h post-hypoxia could be mediated by the concomitant overexpression of HIF-1α as previously reported (Kaul, Fabry, Suzuka, & Zhang, 2013).

4 | DISCUSSION

The present study compared the effects of 4 h of hypoxia followed by short (2 h) or long (6 h) recovery on the Nrf2 pathway in the kidney of sickle cell SAD mice and control C57 mice. Our results showed in normoxia higher renal oxidative stress, HIF-1α and nuclear Nrf2 protein expression in SAD mice. This suggests specific physiological features of the kidneys of SAD mice. In response to H/R stimuli, Nrf2 protein expression and HO-1 transcripts are only modified in SAD mice, with more marked responses in the medulla *vs.* cortex (Figure 4). These responses to H/R stimuli were not associated with greater kidney injury in SAD mice (data not shown) and could suggest a more appropriate response of SAD mouse kidney to H/R stimuli as compared to C57 mice.

4.1 | Renal redox status of SAD mice

In normoxia, the higher lipid peroxidation in SAD compared to C57 mice is consistent with the results of Nath et al. (2001) that demonstrated higher thiobarbituric acid-reactive substance in another model of sickle mice (S+S Antilles) compared to controls. Interestingly, these authors suggested that increased content of haem, a strong pro-oxidant species, could contribute to oxidative stress in transgenic sickle mice. It could also be hypothesized that other means of ROS generation may explain the higher oxidative stress in the kidneys of SAD mice, such as auto-oxidation of sickle RBCs themselves (Hebbel, Eaton, Balasingam, & Steinberg, 1982; Hofstra, Kalra, Meiselman, & Coates, 1996; Sultana, Shen, Rattan, Johnson, & Kalra, 1998), episodes of I/R in the medullary circulation (Pham, Pham, Wilkinson, & Lew, 2000) or activating xanthine oxidase (Chirico & Pialoux, 2012). In addition, HIF-1α staining and HIF target gene expression were significantly higher in normoxia in SAD than in C57 mice. This finding is in agreement with a previous study which reported that the higher oxidative stress in Berkeley mice, a homozygous knockout model of SCD (*vs.* C57), could potentially induce expression of HIF-1α in the cremaster muscle (Kaul et al., 2013).

The higher nuclear Nrf2 protein expression in the medulla and greater levels of SOD, GPX and catalase activities in normoxia, in SAD

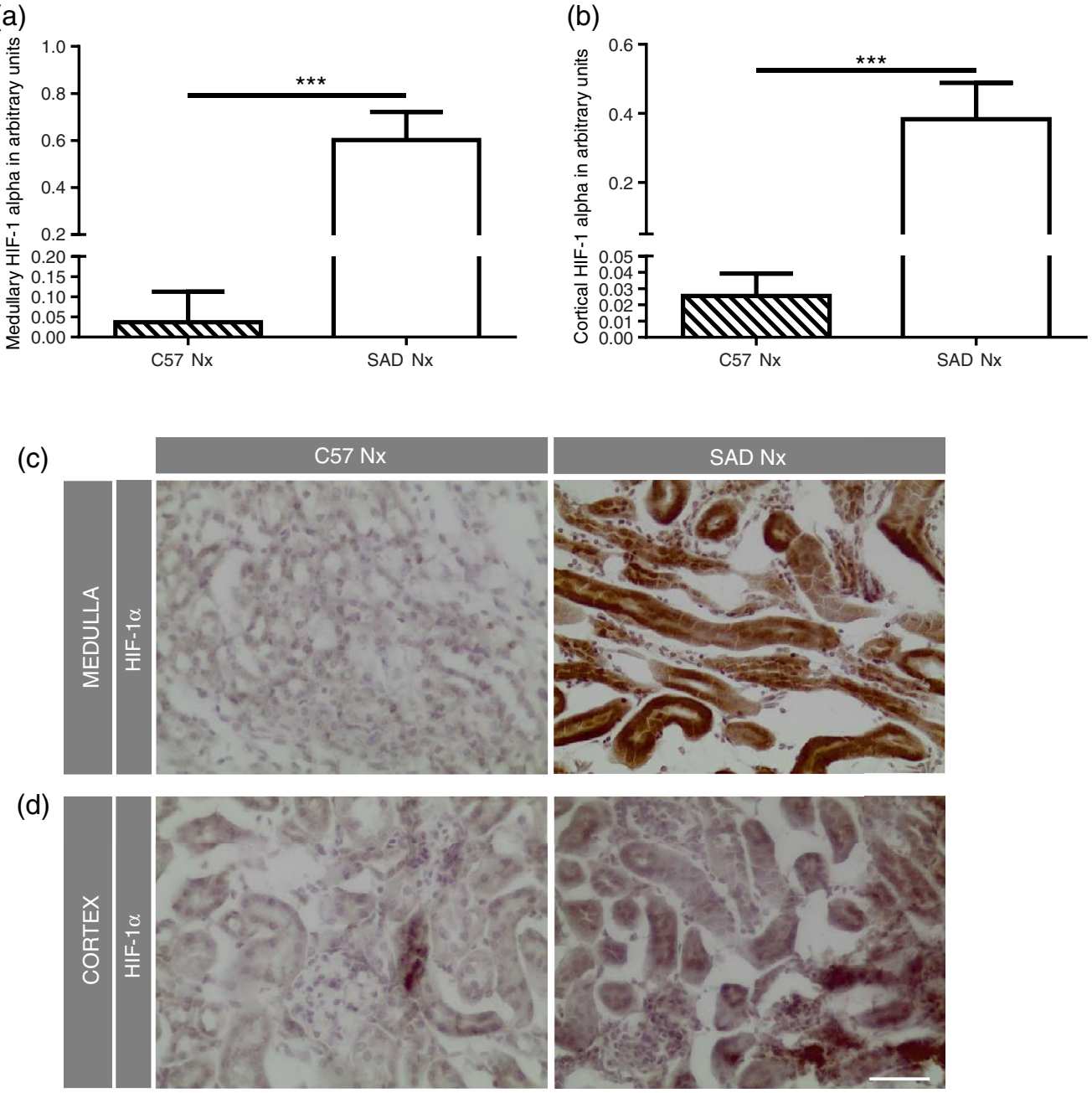

FIGURE 2 HIF-1α immunostaining and HIF-1α-inducible enzymes in the kidney of C57Bl/6J (C57) and sickle cell SAD mice under normoxia ($C57_{Nx}$ and SAD_{Nx}). (a,b) HIF-1α immunostaining scores in the medulla (a) and in the cortex (b) of C57 and sickle cell SAD mice under normoxia (Nx). (c,d) Representative staining for HIF-1α in the medullary (c) and cortical (d) kidney sections. Magnification ×40; scale bar: 50 μm. (e–h) Endothelin-1 (ET-1) (e), inducible nitric oxide synthase (iNOS) (f), vascular endothelial growth factor-A (VEGF-A) (g), and erythropoietin (EPO) (h) mRNA levels in normoxia (Nx). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$: significant difference between C57 and SAD in normoxia. $C57_{Nx}$ and SAD_{Nx}: $n = 10$ per group

vs. C57 mice corroborate the Nrf2-regulated antioxidant pathway for these three enzymes (Kang et al., 2005; Zhang, Chae, Lee, & Hyun, 2012). The higher antioxidant SOD, GPX and catalase activities in SAD *vs.* healthy mice confirm previous observations (Franco, Odom, & Rando, 1999; Zhou, Johnson, & Rando, 2001) and could result to a redox protective mechanism induced by chronic oxidative stress. Indeed MDA-generated stimulation was shown to trigger antioxidant enzyme activation (Ji, Gomez-Cabrera, & Vina, 2006; Radak, Chung, & Goto, 2005).

In summary, our data obtained on oxidative stress markers and HIF-1α support the hypothesis that the kidneys are organs that have increased oxidative stress, in SAD mice (De Paepe & Trudel, 1994; Nath et al., 2001), regardless of the conditions (i.e. normoxia or H/R).

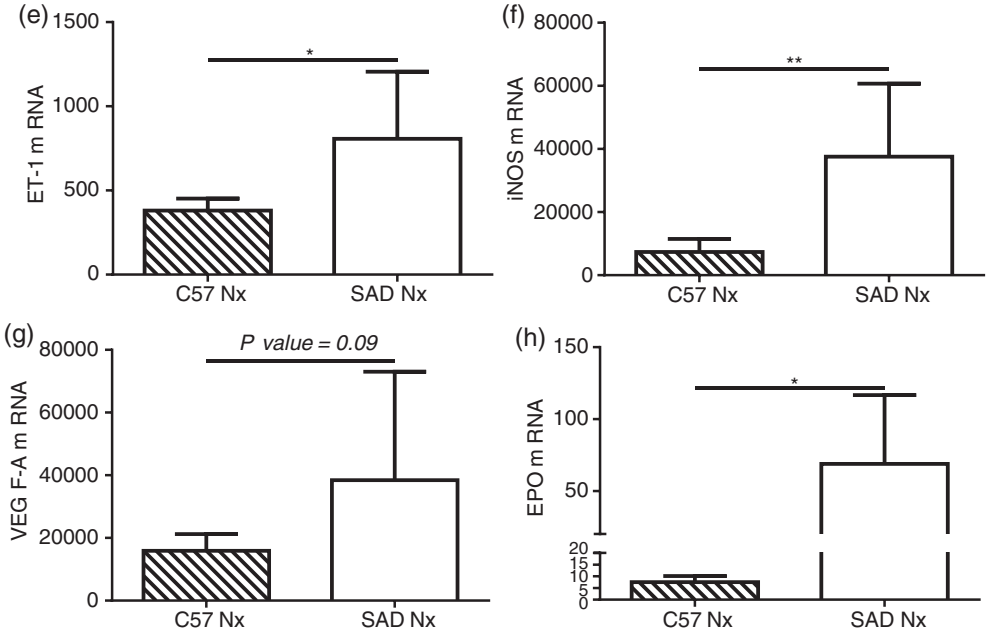

FIGURE 2 Continued

It is also interesting to underline that the increase in Nrf2 signal in the medulla of SAD kidneys after H/R stimulus occurs not only in the nucleus but also in the cytosol (Figure 1c). This cytosolic increase in Nrf2 may result from its transcriptional up-regulation via inflammatory stimuli and NFκB (Tonelli, Chio, & Tuveson, 2018) since renal I/R was reported to induce systemic inflammation in SAD mice (Nath et al., 2005).

4.2 | Effect of hypoxia–reoxygenation time on nuclear Nrf2 protein expression in the kidney of SAD mice

Our results showed that nuclear Nrf2 protein expression decreased only after 4 h of hypoxia and 2 h reoxygenation in the medulla of SAD mice (Figure 1a,c). This result is surprising since ROS are known to inhibit the degradation of Nrf2 by Keap1 and activate its translocation in the nucleus (Leonard et al., 2006); however, in our study, short and long recovery responses of renal oxidative stress were not different compared to normoxia in SAD mice (Table 1). Thus, the decrease of Nrf2 at 2 h post-hypoxia might be explained by the activation of an inhibitory pathway. Because Siah2 is known to be involved in the hypoxia-activated pathway including notably HIF-1α regulation (Nakayama et al., 2004), which increased at 2 h post-hypoxia, we may hypothesize that Siah2 could down-regulate Nrf2 in the kidneys of SAD mice as it was previously demonstrated in non-sickle human embryonic kidney cells (Baba et al., 2013). Also, the lack of changes in the cortex compared to the medulla could be link to the inhomogeneity of renal blood flow and oxygen supply in the whole kidney. The low P_{O_2} and the high metabolic rate in the renal medulla (Epstein, 1997) may increase ROS production and trigger subsequent Nrf2 activation. Meanwhile, a recent study reported that the mitochondria of medullary cells were more efficient and adapted to a hypoxic environment than those in the cortex (Schiffer, Gustafsson, & Palm, 2018).

4.3 | Effect of hypoxia–reoxygenation time on HO-1 mRNA levels in the kidney of SAD mice

Surprisingly, we observed that renal HO-1 transcripts dramatically increased 2 h after reoxygenation (Fig 1e) and returned to normoxia values at 6 h post-hypoxia, while nuclear Nrf2 followed the opposite way. These results contrast with evidence supporting that Nrf2 regulates the mouse HO-1 gene (Sikorski et al., 2004). Nevertheless, Pullarkat et al. (2014) have recently showed that proteasomal inhibition of Nrf2 degradation did not significantly increase mRNA expression of HO-1 in the peripheral blood mononuclear cells culture of SCD patients, suggesting that Nrf2 could play a minor role in regulation of HO-1. Furthermore, as underlined by Ghosh et al. (2011), the up-regulation of HO-1 expression in sickle mice is likely variable according to the organ and the context. Thus, we may hypothesize that HO-1 mRNA activation by H/R in SAD kidneys could be induced by others transcription factors than Nrf2 (Sikorski et al., 2004). In response to hypoxic stress, HIF-1α has been demonstrated to mediate transcriptional activation of the HO-1 gene mainly because the HO-1 gene has a functional HIF binding site (Lee et al., 1997; Yang & Zou, 2001). Our immunohistological results are in line with these latter observations since HIF-1α staining increased in the medulla and in the cortex of SAD mice only after 2 h of reoxygenation and decreased at 6 h post-hypoxia. Thus, it seems that SAD mouse kidney responded appropriately to H/R stimuli as compared to control mice. Based on a recent study (Rossi et al., 2019) which reported that hemin-induced

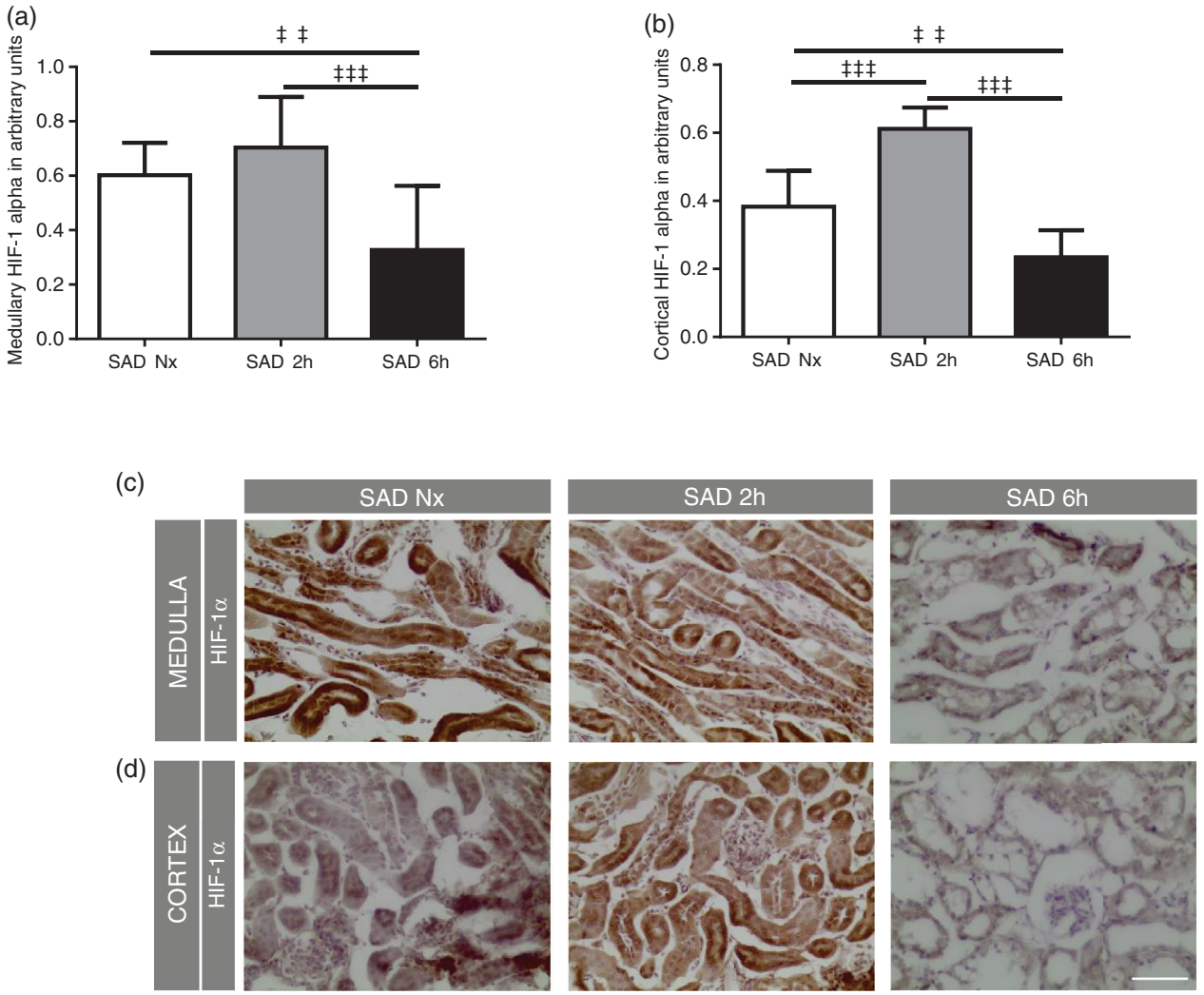

FIGURE 3 HIF-1α immunostaining and HIF-1α-inducible enzymes in the kidney of sickle cell SAD mice in normoxia (SAD Nx) and after 4 h of hypoxia followed by 2 h (SAD 2h) or 6 h (SAD 6h) reoxygenation. (a,b) HIF-1α immunostaining scores in the medulla (a) and in the cortex (b) of C57 and sickle cell SAD mice after 2 h or 6 h reoxygenation. (c,d) Representative staining for HIF-1α in the medullary (c) and cortical (d) kidney sections. Magnification ×40; scale bar: 50 μm. (e–h) Endothelin-1 (ET-1) (e), inducible nitric oxide synthase (iNOS) (f), vascular endothelial growth factor-A (VEGF-A) (g), erythropoietin (EPO) (h) mRNA levels after 2 h or 6 h reoxygenation. $^{\dagger\dagger\dagger}P < 0.001$: significant difference compared with SAD_{2h}. $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$: significant difference compared with SAD_{Nx}. *N* values per group: SAD Nx: 10; SAD 2 h: 10; SAD 6 h: 10

HO-1 prevents acute kidney injury, we may hypothesize that the higher levels of total plasma haem in SAD *vs.* C57 mice (Merle et al., 2018) at basal state could explain the more appropriate response to stress in SAD mice.

Although original, our conclusions are based on associations, and thus further mechanistic studies are needed to confirm the regulation pathway between HIF-1α and HO-1 transcripts in the kidney of SAD mice under H/R stimuli.

5 | CONCLUSIONS

To conclude, in this study, we showed that SAD mice expressed modified renal oxidative stress and molecular adaptation to hypoxia. In addition, our results suggest that HO-1 transcript up-regulation may occur independently of Nrf2 protein expression in the SAD mouse model. This up-regulation could involve another regulatory pathway induced by hypoxia including HIF-1α (Figure 4).

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COMPETING INTERESTS

The authors report no conflict of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included in this article.

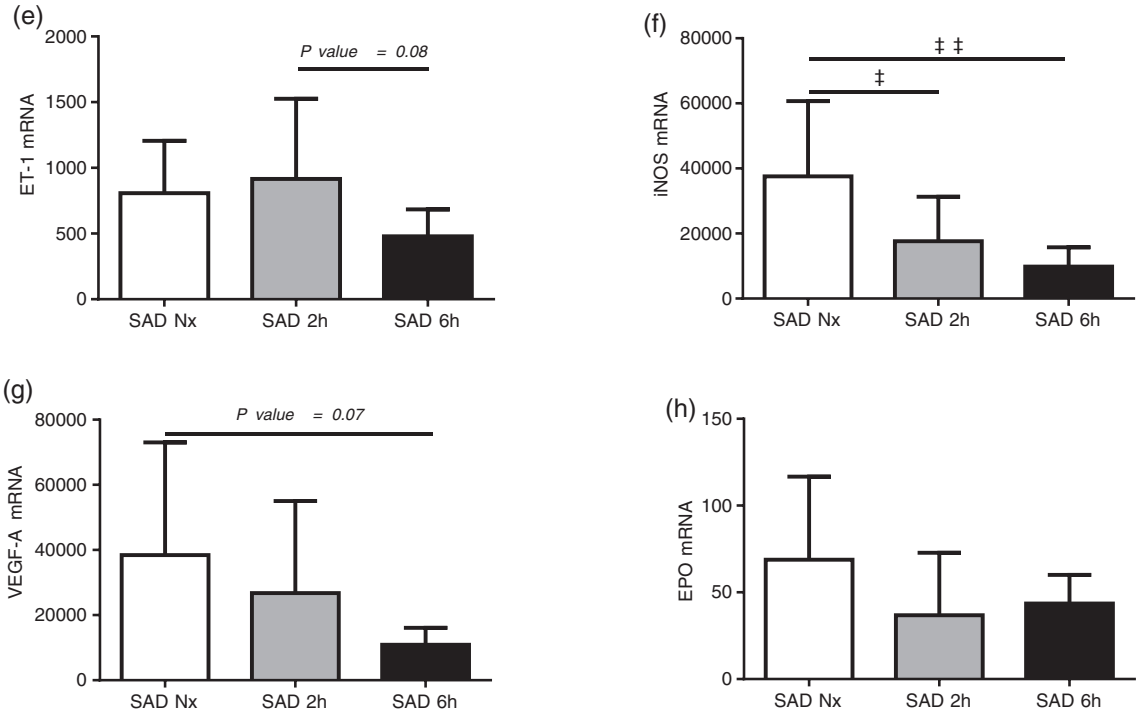

FIGURE 3 Continued

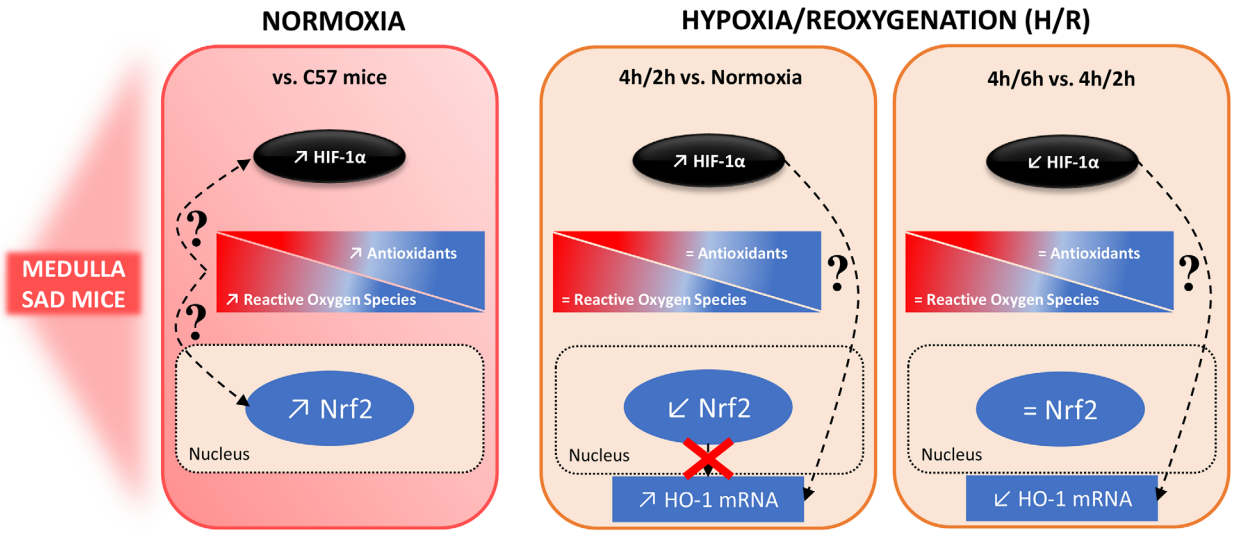

FIGURE 4 Illustration of hypothetical links between oxidative stress, Nrf2 and HIF-1α pathways in normoxia and under hypoxia–reoxygenation (H/R) stimuli in the renal medulla of transgenic SAD mice. In normoxia, according to our results, SAD mice expressed higher medullary oxidative stress compared to healthy mice (C57), which could explain the increase in nuclear Nrf2 and HIF-1α protein expression. In contrast, in SAD mice, while medullary oxidative stress did not change after H/R, independent of the duration of reoxygenation period (2 h, 6 h), nuclear Nrf2 protein expression decreased after 2 h post-hypoxia. However, we did not find a link between Nrf2 and the transcriptional regulation of HO-1 at this time point. Thus, we may hypothesize that the up-regulation of HO-1 transcripts after 2 h post-hypoxia may involve HIF-1α known to be implicated in HO-1 regulation under hypoxia (Lee et al., 1997; Yang & Zou, 2001). Similarly, the down-regulation of HO-1 transcripts after 6 h post-hypoxia may also be explained by the decrease of HIF-1α protein expression at this time point. HIF-1α, hypoxia inducible factor-1 alpha; HO-1, haem oxygenase1; Nrf2, nuclear factor erythroid 2-related factor 2. Dashed arrows represent hypothetical links

AUTHOR CONTRIBUTIONS

Experiments were performed at the LIBM EA7424; the Institut NeuroMyogène (UMR5310-INSERM U1217), University of Lyon 1, France and the Department of Medicine, University of Verona and AOUI-Verona. Conception or design of the work: C.F., C.M. and V.P. Acquisition, analysis or interpretation of data for the work: C.F., G.J., E.A., T.D., E.C., P.C., P.M., A.M., L.F. and V.P. Drafting of the work, or

revising it critically for important intellectual content: C.F., G.J., E.A., T.D., E.C., P.C., P.M., A.M., L.F. and V.P. All authors approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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